UNIVERSITETET I OSLO
DET MEDISINSKE FAKULTET

STUDIES ON SUBFRACTIONS OF FIBRINOGEN

With special emphasis on fibrinogen quantification, viscosity and inflammation

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I dedicate this thesis to the memory of Hans Christian Godal.

Oslo, February 2008

Torstein Jensen

2. ABBREVIATIONS

aa	amino acids
αC	carboxy terminal end of the fibrinogen α-chain
ADP	adenosine diphosphate
CHD	coronary heart disease
CRP	C-reactive protein
EDTA	ethylene-diamine-tetra-acetic acid
FgDP	fibrinogen degradation products
FnDP	fibrin degradation products
FPA	fibrinopeptide A
FPB	fibrinopeptide B
HMW	high molecular weight
IHD	ischemic heart disease
IL-1	interleukin 1
IL-6	interleukin 6
ISTH	international society of thrombosis and haemostasis
kDa	kiloDalton
LMW	low molecular weight
LMW`	very low molecular weight
LPS	lipopolysaccharid
M	molar
mPa.s	millipascal seconds
MW	molecular weight
N-terminal	amino-terminal
PAGE	polyacrylamid gel electrophoresis
PAI	plasminogen activator inhibitor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate

SD	standard deviation
TAFI	thrombin-activatable fibrinolysis inhibitor
TF	tissue factor
TNF	tumor necrosis factor
U-PA	urokinase-type plasminogen activator
U	unit
vWF	von Willebrand Factor

3. LIST OF PAPERS

PAPER I

Influence of freeze-drying on the clotting properties of fibrinogen in plasma.
Torstein Jensen, Sigrun Halvorsen, Hans C. Godal, Ole H. Skjønsberg
Thrombosis Research 2002; 105: 499– 502.

PAPER II

Discrepancy between fibrinogen concentrations determined by clotting rate and clottability assays during the acute-phase reaction.
Torstein Jensen, Sigrun Halvorsen, Hans C. Godal, Per M. Sandset and Ole H. Skjønsberg. Thrombosis Research 2000; 100: 397-403.

PAPER III

The viscosity of fibrinogen subfractions and of EDTA denatured fibrinogen do not differ from that of native fibrinogen.
Torstein Jensen, Sigrun Halvorsen, Hans C. Godal, Per M. Sandset, Ole H. Skjønsberg. Thrombosis Research 2004; 113: 51–56.

PAPER IV

A daily glass of red wine induces a prolonged reduction in plasma viscosity: a randomized controlled trial.
Torstein Jensen, Lars J. Retterstøl, Per M. Sandset, Hans C. Godal, Ole H. Skjønsberg. Blood Coagulation and Fibrinolysis 2006; 17: 471–476.

PAPER V

Fibrinogen and fibrin induce synthesis of proinflammatory cytokines from isolated peripheral blood mononuclear cells.
Torstein Jensen, Peter Kierulf, Olav Klingenberg, Gunn Brit Joø, Hans C. Godal, Per M. Sandset, Ole H. Skjønsberg.
Thrombosis and Haemostasis 2007; 20: 822-829.

4. GENERAL INTRODUCTION

Fibrinogen is a large, soluble glycoprotein. It plays a central role in human hemostasis through its conversion to fibrin in the last step of the coagulation cascade, and by its essential role in platelet aggregation. Normal plasma concentration is 1.7-4.0 g/l. Being an acute-phase protein, the concentration rises rapidly during inflammatory conditions, major surgery and certain cancer forms (1-3). The plasma concentration results from a complex interaction between genetic and environmental factors (4). Plasma fibrinogen levels are higher in females than in males, and increased concentrations are found in postmenopausal women, smokers, patients with diabetes, obesity, hypercholesterolemia and hypertension (5). Factors which may decrease fibrinogen levels include physical exercise, weight loss, moderate alcohol consumption and smoking cessation (4;6-10). Treatment with fibrates and platelet aggregation inhibiting drugs such as ticlopidine may lower plasma levels of fibrinogen (11). Different responses of hormone replacement therapy have been reported (9;12-14). Genetic heritability accounts for about 50% of the variance in fibrinogen concentration between individuals (15).

Structure

In 1959 Hall and Slayter produced electron microscopy images of fibrinogen revealing a rod-like molecule with 3 interconnected globules (16). The length was estimated to be 475 ± 25 Å. The diameter of the center globule was approximately 50 Å, and the distal globules were approximately 65 Å (16;17). The structure was later confirmed by other investigators (18;19). Using atomic force microscopy, Marchant and colleagues obtained three dimensional images of fibrinogen molecules under aqueous conditions, showing a mixture of monomers, dimers and trimeric structures linked through D-D globular interconnections (20).

The fibrinogen molecule is a dimer consisting of 3 pairs of the non-identical polypeptide chains Aα, Bβ and γ with molecular weight 340 kDa (21). The nomenclature $(A\alpha,B\beta,\gamma)_2$ reflects the peptides A and B cleaved from fibrinogen by thrombin and the longer α-, β- and γ-chains. No peptides are split from the γ-chain during the conversion of fibrinogen

to fibrin. The total number of amino-acids is 2964, and the complete amino-acid

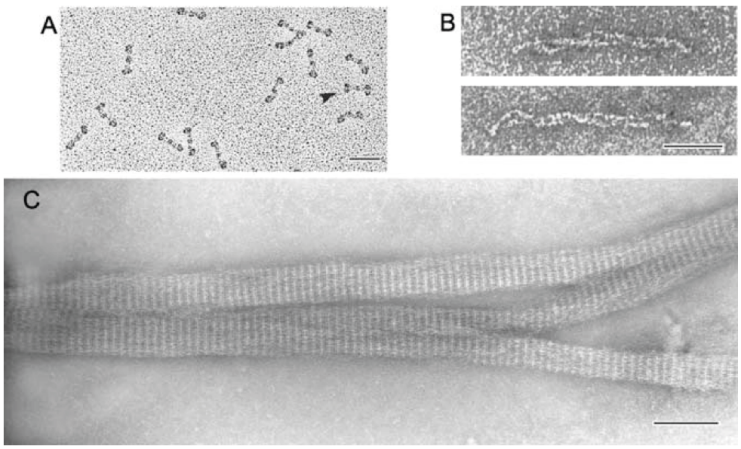

Figure 1. Electron microscope images showing fibrinogen molecules with a central (E)- and two peripheral (D) regions (1A), fibrin protofibrils with half-staggered overlapping fibrin monomers (1B), and thicker, branching fibrin fibers (1C). Reproduced from Weisel (22).

sequence was presented in 1980 by Henschen and Lottspeich (23;24). The molecular weights of the individual chains are 66.5 kDa, (610 amino-acids), 52 kDa (461 amino-acids), and 46.5 kDa (411 amino-acids) for the Aα-, Bβ- and γ-chain, respectively (25;26). The 6 polypeptide chains are gathered in the central E region (E-domain) (27), which is connected to the 2 D regions (D-domains) of the molecule by the "interconnector", comprising 111-112 amino acids of the Aα-, Bβ-, and γ-chains joined together in a coiled-coil (28) (figure 2). The D region can be further divided into a distal nodule containing the C-terminal part of the γ-chain (γC), and the proximal nodule including the C-terminal Bβ-chain. The C-terminal part of the Aα-chain (αC) consists of an elongated polypeptide extending from the D region, interacting with the central E region, and forming intra-molecular αC-αC connections (29;30).

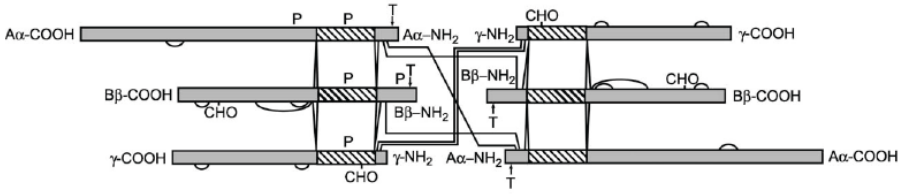

Figure 2. Schematic drawing of the fibrinogen molecule showing the N- and C-terminal ends of the Aα-, Bβ-, and γ-chains. The coiled-coil regions are illustrated with diagonal lines, and disulfide bonds marked with solid lines. CHO indicates carbohydrate attachment sites, and P and T represent major cleavage sites for plasmin and thrombin, respectively. Adapted from Hantgan (31).

Calcium

Calcium is important for the structural integrity and function of the fibrinogen molecule. High affinity binding sites for calcium are located in the γ-chain at Asp^{318}, Asp^{320}, Gly^{324} and Phe^{322}. In addition several low affinity calcium binding sites exists which are less well defined (32). In plasma the binding sites are usually occupied providing protection from enzymatic degradation (33;34).

Disulphide bonds

Twenty nine disulfide chains located in the Aα- (8), Bβ- (11) and γ-chains (10) hold the 6 chains together (35). The central E-region contains the 2 pairs of A and B peptides linked together by three inter-chained disulfide bonds, 1 between the Aα-chains and 2 between the γ-chains. One disulfide bridge links the Aα- and the Bβ-chain.

Carbohydrate chains

Fibrinogen is a glycoprotein containing four oligosaccharides located at $B\beta^{364}$ and γ^{52} and linked through N-glycosyl bonds. The α-chain contains no carbohydrate side chains (36). The carbohydrate chains are of major importance for the solubility of fibrinogen, and modifications of the carbohydrate chains affect the fibrin polymerization properties and clot structure. Fibrinogen contains equal amounts of mono- and disialicated chains, and variations of the sialic-acid content of the carbohydrate chains account for some of the

heterogeneity of fibrinogen in plasma. Complete deglycosylation results in accelerated fibrin polymerization, increased lateral aggregation with thicker, less branched fibrin fibers and a more porous network (37).

Synthesis of fibrinogen

Fibrinogen in plasma is mainly produced in the liver (38;39) with a half-life in plasma of 3-5 days (40-42). Synthesis under normal conditions range from 1.7-5 g/day (43). Fibrinogen is an acute-phase protein, and during acute-phase conditions such as major surgery, bacterial infections and trauma, gene transcription and release of fibrinogen rises rapidly. Cytokines in the interleukin-6 (IL-6) family have been recognized as important stimulants to increased fibrinogen synthesis. Plasma fibrinogen levels can, thus, increase manifold. Fibrinogen and fibrin degradation products (FgDP/FnDP) have been shown to stimulate fibrinogen synthesis in vitro (44;45). The results are inconsistent, however, and the physiological role of FgDP and FnDP on fibrinogen synthesis in vivo is not clear (44-48). Although 75% of the total fibrinogen is found in plasma, it may also be found in lymph nodes, blood platelets and in interstitial fluid. In vitro studies have shown that fibrinogen may also be synthesized by non-hepatic cell lines such as epithelial cells from lung and intestine as well as trophoblasts and granulosa cells (49-52). The physiological role of this locally produced fibrinogen is not clear. Although the Aα, Bβ, and γ chains are encoded by dedicated genes closely linked on the distal third of the long arm of chromosome 4, bands q23-32 (53), the transcription of the 3 different genes are closely coordinated (54). The gene for the Aα-chain, located at the centre of the fibrinogen cluster, consists of 5 exons that translate into 625 amino acids, 15 of which are removed after transcription. The gene coding for the Bβ-chains contain 8 exons and codes for a 461 residue polypeptide and the gene coding for γ-chain comprise 10 exons coding for the 411 residue γ –chain (53).

The intracellular assembly of the 3 chains in cytoplasmatic reticulum joins single chains to a 3-chained half-molecule which is subsequently joined to the final dimeric fibrinogen molecule. When hepatocytes are stimulated with IL-6, the genes for the 3 subunits are upregulated simultaneously and to the same extent (55;56). Cytokines such as

interleukin-1β and tumor necrosis factor-α and glucocorticosteroids may modify the IL-6 response (54;57).

Variations of the α-chain

Heterogeneity in circulating fibrinogen molecules are dominated by heterogeneity of the C-terminal α-chain (58;59). Depending on the degree of C-terminal α-chain degradation, 3 main fibrinogen fractions have been described: High molecular weight (HMW-) fibrinogen (MW 340 kDa) having both its α-chains intact; low molecular weight (LMW-) fibrinogen (305 kDa), which has lost the C-terminal end of one α-chain, and very low molecular weight (LMW`-) fibrinogen (280 kDa), which has lost the C-terminal end of both α-chains (2;60). These fractions constitute approximately 70%, 25% and 5%, respectively, of the total plasma fibrinogen content. Experiments using western-blotting and immunostaining techniques have, however, revealed a considerable larger diversity of Aα-chain remnants with intact N-terminal ends and MW 66,200 to 36,000 Da in normal plasma (61). High molecular weight fibrinogen has lower solubility, shorter thrombin clotting time and increased clottability compared to the low molecular forms, illustrating the importance of the carboxy- terminal α-chain for the clotting properties of fibrinogen (62-68). Furthermore, clots from αC-degraded fibrinogen have decreased turbidity and lack mechanical strength compared to native fibrin clots. Although not essential for clot formation, the loss of one or both αC-terminals significantly retards the polymerization process (29;69). In 1992 an extended version of the α-chain, αE, was described (70;71). Based on predicted mass, fibrinogen molecules containing the 236 residue extended α-chains are called Fibrinogen 420 (MW 420 kDa), to distinguish it from the more predominant Fibrinogen 340. Fibrinogen 420 comprises approximately 1 % of the total fibrinogen concentration in adults (72). It has been shown to be involved in leukocyte adhesion and migration through binding of the α_EC terminal to the leukocyte integrins $\alpha_M\beta 2$ and $\alpha_X\beta 2$ (73).

Variations of the γ –chain

Alternative processing of the γ-chain gene transcript gives rise to an alternative γ-chain, γ`, with a negatively charged 20 amino acid extension at the C terminal end of the γ-chain, substituting the γ 408–411 with a 20 amino acid sequence, γ`408–427, that contains two sulfated tyrosines. The γ`-chain constitutes about 8% of the γ chains (74-76), and in plasma 10-15% of the fibrinogen molecules contain the γ` variant (77). The γ`-chain includes binding sites for thrombin and the B chain of factor XIII, suggesting that the γ`-chain function as a carrier for factor XIII in the circulation, making it readily accessible during fibrin formation (78). The γ- and γ`-chains are equally effective cross linked by XIIIa. Due to lack of the full platelet binding sequence 400-411, the γ`-chain does not support ADP induced fibrinogen mediated platelet aggregation (79;80).

Conversion of fibrinogen to fibrin

The conversion of soluble fibrinogen to an insoluble fibrin network is initiated by the thrombin mediated removal of the fibrinopeptides A and B from the N-terminal Aα- and Bβ-chains (81). The cleavage of FPA at amino acids 15-16 occurs more rapidly than the split of FPB between amino acids 16-17 (82), which has been suggested to be involved in lateral growth and branching of the fibrin polymer (83). Thus, 2 FPA and 2 FPB are released from each fibrinogen molecule exposing the peptide sequence Gly-His-Arg-Pro-amide in the α-knob, and the Gly-Pro-Arg-Pro-amide from the β-knob. Complimentary "holes" are located in the globular C-terminal β- and γ-chains. Although structurally similar, the βC- and γC-holes are able to distinguish between these peptides.

Thrombin

Thrombin is a serine protease produced in the liver as the 70 kD zymogene prothrombin (84). The 579 residue prothrombin undergoes posttranscriptional modifications before it is secreted into the circulation. Following damage to the blood vessel and exposition of subendothelial tissue factor (TF), factor X is converted to Xa in the presence of FVIIa and Ca^{2+} in the initial phase of coagulation. Prothrombin is subsequently converted to the 35 kDa biologically active thrombin by the prothrombinase complex, including FVa, FXa, and anionic phospholipids in the presence of Ca^{2+}. Thus, prothrombin is cleaved

after Arg^{320} forming the intermediate mezothrombin and subsequently after Arg^{271} to form thrombin (85). Thrombin comprises a 49 residue light chain and a 259 residue heavy chain linked together by a single disulphide bridge. The heavy chain is intra-connected through 3 disulphide bonds. The initial small amounts of thrombin formed by the extrinsic pathway trigger the intrinsic coagulation pathway through activation of FXI, FVIII and FV, thus, creating more thrombin.

The activity half-life of thrombin is only 14 seconds, and it is rapidly inactivated by inhibitors. The principal circulating thrombin inhibitors are antithrombin, heparin cofactor II (HCII) and protease nexin 1 (86;87). However, thrombin also has the ability to inhibit its own generation through interaction with endothelial bound thrombomodulin, which activates the protein C pathway. Protein C and protein S effectively inhibit factors Va and VIIIa, thus preventing further thrombin generation. The proteolytic properties of thrombin are affected through interaction with Na^+. Several Na^+ binding sites have been identified, the principal binding sites being confined to the C-terminal region of the heavy chain (88;89). Binding of Na^+ to thrombin alters the protein structure and allosterically enhances its catalytic properties. At physiological Na^+ concentrations, the sodium binding sites in thrombin are fully occupied.

Polymerization

The removal of the acidic FPA from fibrinogen changes the N-terminal net charge, and is followed by a spontaneous polymerization step in which two fibrin monomers (des-AA fibrin) are arranged in a non-covalent bonded staggered overlap between the E-domain and complimentary binding sites in the C-terminal γ-chain of the D-domain (90). In addition to the E-/D-domain interaction, D/D-domain intermolecular bonds form in the fibrin oligomer, thus forming a double stranded protofibril. The release of fibrinopeptide B, although starting at the same time as the release of FPA, occurs at a slower rate than the release of FPA (82;91). Fibrin gel formation, however, accelerates the FPB release rate about sevenfold (92;93). The removal of FPB exposes an additional polymerization site in the E-domain called B “knobs” which are complementary to b “holes” in the C-terminal β-chain (94;95). Removal of the two FPBs induces a conformational change of the α-chain. The two α-chains, usually stretching parallel to the coiled coil and meeting in

the central E-domain, dissociate and form a mobile extension from the D-domain. This allows interaction between neighboring α-chains, which promotes lateral aggregation of fibrin protofibrils and strengthening of the mechanical properties of the clots (96). The non-covalent ligation between the E-/D-domains are easily broken by solvents such as urea, acetic acid and sodium bromide. Active "knobs" in fibrin monomers or fibrin protofibrils may bind to always exposed "holes" in circulating fibrinogen forming soluble fibrinogen-fibrin complexes (97;98), thus, inhibiting fibrin polymerization. Even degradation products of fibrin and fibrinogen may interact with "holes" in the D-domain and affect clot formation

The fibrin clot architecture is influenced by several factors. High fibrinogen levels and fast activation leads to thin fibrin fibers and more rigid clots with smaller pores, making it less susceptible to fibrinolytics. Slow activation, on the other hand, leads to thicker fibrin strands with larger pores (99). Ionic strength, increased glucose or homocysteine levels, the presence of other plasma proteins (100) and cross linking of proteins such as PAI-2, TAFI, α-2 antiplasmin as well as fibrinogen- and factor XIII-polymorphisms may all affect the fibrin clot structure (for review see (101)).

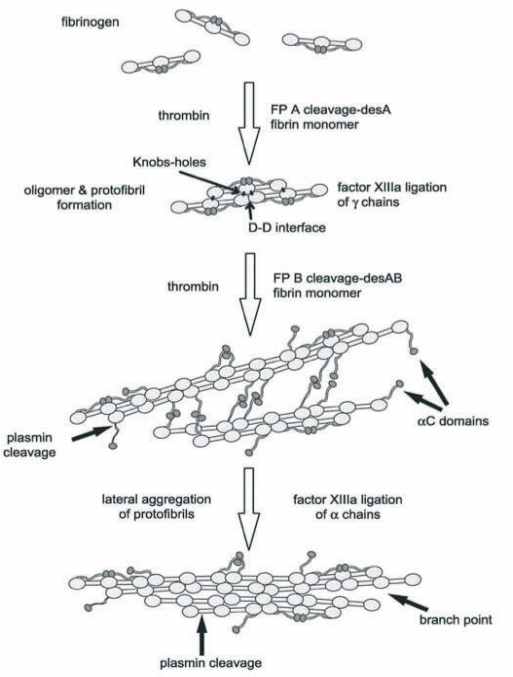

Figure 3. Illustration of the conversion of fibrinogen to fibrin by thrombin, fibrin polymerization and factor XIIIa mediated cross-linking according to Weisel (22).

Cross-linking

The fibrin matrix formed by the action of thrombin is fragile. Through factor XIIIa mediated cross-linking of the γ-chains the fibrin clot is strengthened and made resistant to physical stress and proteolytic attacks. Factor XIII in plasma is a 326 kDa tetramer including 2 A chains and 2 B chains. About half of the total factor XIII in blood is contained in platelets in the form of A2 dimers. In plasma, most of the factor XIII zymogen A2B2 is bound to fibrinogen, especially to the γ`-chain of Fibrinogen 420 (102). Factor XIII is activated through the thrombin mediated proteolysis of the A chain. Activated factor XIII induces inter-chain covalent cross-linking between one C-terminal glutamine (γGln398/399) and one lysine (γLys406) residue in adjacent γ-chains (103;104). Due to the close proximity of the acceptor and donor sites in the fibrin clot,

factor XIIIa catalyses cross-linking of fibrin much faster than cross-linking of fibrinogen (105). Intermolecular α-chain cross-linking generating α-oligomers and polymers occur at a slower rate and facilitate lateral fibril association and fiber growth. Crosslinking may also occur between fibrin α- and γ-chains, and even between fibrinogen γ-chains.

Metabolism of fibrinogen and fibrin

Only 2-3% of the fibrinogen molecules are removed from the circulation as a consequence of coagulation and subsequent fibrinolysis (106). Although plasmin mediated proteolysis is the dominating fibrin degradation pathway, plasmin does not play an important role in the degradation of fibrinogen, except during exogenous treatment with plasminogen activators (107;108). Furthermore, it seems that HMW-fibrinogen only partially is metabolized to LMW-fibrinogen in vivo (108-110). To what extent HMW-fibrinogen is metabolized to LMW`-fibrinogen, and which additional mechanisms are involved in the degradation of fibrinogen in vivo are not clear.

In vitro studies have shown that the Aα-, Bβ-, and γ-chains differ in their susceptibility to plasmin (111). The Aα-chain, with its elongated C-terminal end, is more vulnerable to plasmin attacks than the Bβ- and γ-chains. Similarly, the Bβ-chains are more susceptible to plasmin degradation than the γ-chains. The destruction of the αC-domain results in the formation of fragment X (MW 250 kDa) and degradation products from the α-chain named A, B and C (111;112). Further digestion of the coiled-coil of the fragment X, gives rise to fragments D (MW 83-100 kDa) and Y (155 kDa). Fragment Y is quickly degraded to the terminal fragments D (81 kDa) and E (41 kDa) (figure 4). Fragment E corresponds to the N-terminal domain of the native fibrinogen molecule. Fragments D and E constitute the final plasmin mediated fibrinogen degradation products. The affinity of plasminogen to polymerizing fibrin is stronger than to circulating fibrinogen. Although fibrinogen has high affinity binding sites for plasminogen at the C-terminal α-chain, the formation of the ternary complex fibrin-plasminogen-t-PA is required to accomplish effective t-PA mediated plasminogen activation. The fibrinolysis of cross-linked fibrin creates distinct degradation products such as D-D (D-dimer), comprising two fragments D joined by γ-γ cross-links (113;114), and the non-covalent combination of fragment D-D with fragment E (115). Further degradation of D-dimer, and digestion of the C-

terminal end of the Aα-chain by human neutrophil elastase has been shown in vitro (64;116).

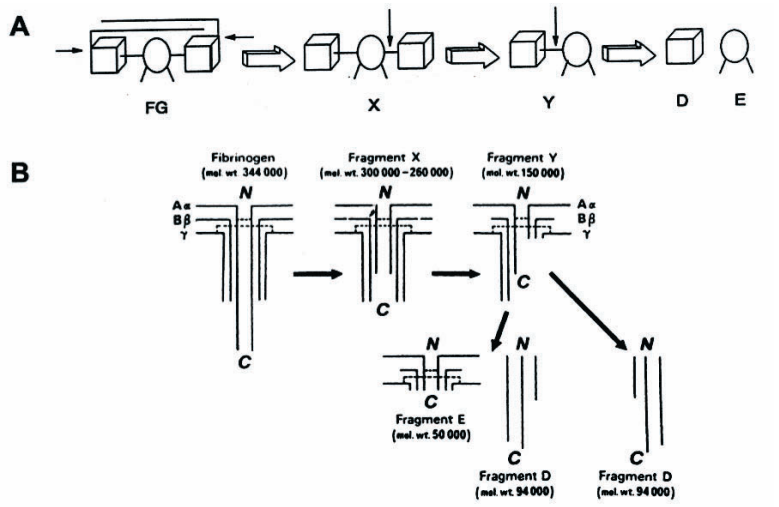

Figure 4. Illustration of the plasmin mediated degradation of fibrinogen to the intermediary X and Y molecules, and the final degradation products D and E. Reproduced from Gaffney (113).

Fibrinogen and risk of cardiovascular disease

A number of clinical trials have shown an association between high fibrinogen levels and increased risk of cardiovascular disease (11;117-120). In a metaanalysis comprising 18 prospective studies and 4000 patients with coronary heart disease (CHD), the relative risk of CHD was 1.8 (95 % confidence interval 1.6-2.0) in patients with fibrinogen concentration in the highest tertile compared to the lowest (121). Increased plasma fibrinogen concentrations have also been related to adverse prognosis following myocardial infarction (122;123), increased risk of stroke (124) and increased cardiovascular- and total mortality in asymptomatic patients (118). The observation that fibrinogen is a strong and independent marker for CHD has raised the question whether

high fibrinogen levels promote the atherothrombotic process, or is only a marker of the chronic low-grade inflammation associated with atherosclerotic vascular disease (125). Possible mechanisms by which fibrinogen may increase CHD risk include its contribution to plasma- (126) and blood viscosity (127;128). Furthermore, a high fibrinogen concentration has been shown to correlate with increased size, weight, and fibrin content of thrombi (129). The key role of fibrinogen in platelet aggregation, and its modulating effect on endothelial function and smooth muscle proliferation, might also affect the atherothrombotic process. On the other hand, studies using genetically modified mice have indicated that fibrinogen is not strictly required for the development of advanced atherosclerotic disease (130).

Fibrinogen and plasma viscosity

Viscosity (η) can be defined as a fluid's resistance to flow attributable to internal friction between molecules or larger particles (131). Isaac Newton (1642-1727) described a model for the viscous properties of fluids, suggesting that the viscosity is proportional to the velocity at which parts of the fluid are separated from one another due to the flow. Newtonian fluids flow like water, and is characterized by changes in its shear rate being proportional equal to changes in shear stress (γ = V/H where V is the velocity and H is the distance between two parallel plates). Shear stress is the applied force per unit area which causes relative movement of fluid layers, and can be described by the equation: $\tau = \mu \frac{dv}{dx}$ (τ= the shear stress exerted by the fluid, μ=the fluid viscosity, dv/dx=the velocity gradient perpendicular to the direction of shear). In non-newtonian fluids, however, the viscosity changes inversely with the shear rate. This implies that for a Newtonian fluid such as plasma, viscosity depends only on pressure, temperature and the chemical composition of the fluid. Different principles have been utilized to determine the viscosity of blood and plasma (132). The "falling-ball" viscometer provides an easy and precise method of measuring the viscosity of Newtonian, low-viscosity fluids such as plasma. By measuring the time taken by a solid sphere to travel a given distance through an inclined glass tube filled with the sample, the viscosity can be calculated.

Albumin, globulins (alpha-1, alpha-2, beta and gamma globulins) and clotting factors constitute the main proteins in plasma. In addition to plasma concentration, the size and shape of proteins determine the effect on plasma viscosity (133). The fibrinogen molecule is a large, rod shaped protein, and fibrinogen has been shown to be a major determinant of plasma viscosity. Variations in plasma viscosity between subjects can partly be attributed to variations in plasma fibrinogen levels (134;135).

Fibrinogen and acute-phase

The initial host response to tissue injury such as infections, major surgery, trauma and some malignant conditions comprises a series of predetermined, coordinated events. This early immune response, called the "acute-phase reaction" (APR), is initiated by the recognition by specific cell receptors of microbial antigens such as lipopolysaccarides (LPS) or other components of the bacterial cell wall (136). The APR includes a local and a systemic response characterized by fever, leukocytosis and increased metabolic rate. In the micro vasculature changes in vascular permeability is observed. Important cytokines involved in the amplification and propagation of the APR include IL-1β, TNF-α, IL-6 and IFN-γ (137). Animal studies have indicated a critical role of IL-6 on upregulation of the synthesis and release of acute phase proteins such as clotting-, and complement factors, immune modulating proteins such as fibronectin, CRP, amyloid A and proteins such as haptoglobin, ferritin, ceruloplasmin and α_1-antitrypsin (137;138). The increased mRNA transcription of the different acute phase proteins is mediated through activation of various nuclear factors (139;140). Plasma fibrinogen concentration may increase 2 to 10 fold during acute phase conditions. Holm and colleagues showed that the increased fibrinogen concentration was mainly due to increased levels of HMW-fibrinogen, with a modest increase in LMW-fibrinogen (2). Due to the increase in HMW-fibrinogen synthesis, the HMW-/LMW-fibrinogen ratio raises 4-8 hours following the initiation of the acute phase, reaching its highest level after 3-4 days (2). The elevated HMW-/LMW-fibrinogen ratio gradually returns to normal levels during the ensuing week, whereas total fibrinogen levels may remain elevated for a longer period of time (2). HMW-fibrinogen has been shown to have increased clotting rate as reflected by shorter thrombin clotting time compared to LMW-, and LMW`-fibrinogen (63). The degree of phosphorylation of

fibrinopeptide A has been shown to increase from 25-30% in normal plasma to more than 50% during acute phase conditions (141).

Fibrinolysis

Activation of coagulation at sites of vascular damage is vital to prevent fatal blood loss. Confining the coagulation process to the place of injury and the subsequent lysis of the clot are, however, equally important to prevent excessive thrombosis and maintain patency of the vasculature. The fibrinolytic system comprises the proenzyme plasminogen, which is converted to the active enzyme plasmin by tissue type plasminogen activator (t-PA) or urokinase type plasminogen activator (u-PA). Plasmin cleaves the fibrin(ogen) molecule at multiple sites resulting in the formation of fibrin(ogen) degradation products (FgDP and FnDP).

Plasminogen

Plasminogen is a single-chain glycoprotein found in plasma at 1.5-2.0 μM concentrations. The molecule comprises 791 amino acids with MW 92 kDa, including 5 homologous triple-loop structures of MW 10 kDa each (142). The interaction of plasmin with fibrin and α_2-antiplasmin is mediated by lysine- and aminohexyl binding sites located in the triple-loops. In addition to the native glu-plasminogen, limited plasmic digestion results in modified forms, lys-plasminogen, containing N-terminal lysine, valine or methionine (143). Through cleavage of the Arg^{561}-Val^{562} peptide bond plasminogen is converted to the active two-chain serine protease plasmin (144). Plasmin is the natural protease for the dissolution of fibrin in vivo.

Plasminogen activators

Tissue type plasminogen activator (t-PA) is a 527 residue serine protease with MW 70 kDa. Hydrolysis of the Arg^{275}–Ile^{276} bond converts it to a two chained form, interconnected by a disulfide bond (145). It is released from the vascular endothelium into the vessel lumen as a result of stress, exercise, venous occlusion and specific drugs (146). Circulating t-PA is mostly bound to plasminogen activator inhibitors (PAI) (147). Residues 276-527 constitute the serine protease domain with the active site amino acids

His ^{322}, Asp^{371} and Ser^{478} (148). t-PA activity is greatly enhanced by the presence of fibrin, and is further amplified through the subsequent formation of fragment-X through plasmin mediated splitting of the C terminal α-chain and N-terminal of the β–chain of fibrin (149). In vitro studies suggest that the plasminogen activating capacity of the 3 natural occurring principal fibrin subfractions with different degree of α-C degradation does not differ (150). Kinetic in vitro studies on the activation of plasminogen in the presence of fibrin(ogen) suggest that t-PA adheres to the fibrin surface with subsequent addition of plasminogen to form a cyclic ternary complex, thus preventing the activation of circulating plasminogen (151). The plasmin formed on the fibrin surface is not readily inactivated by circulating α_2-antiplasmin (half-life 10-100 seconds). Circulating plasmin, on the other hand, has a half-life of only 0.1 seconds (152).

Urokinase-type plasminogen activator (u-PA)

u-PA is synthesized as a single chain (scu-PA) pro-urokinase, which is converted to the active two-chained urokinase-type plasminogen activator (tcu-PA) by plasmin. Even enzymes such as kallikrein, trypsin, cathepsin B, human T cell associated serine proteinase-1, and thermolysin may catalyze the proteolysis of scu-PA. tcu-PA is a 411 residue serine protease with MW 55 kDa containing the active site triad His^{204} , Asp^{255} , and Ser^{356}. The plasminogen activating potential of scu-PA is small compared with tcu-PA. However, the modest plasmin quantity generated by scu-PA is sufficient to stimulate further plasminogen activation by converting scu-PA to the active tcu-PA (153;154).

Ligation of u-PA to its cell-surface receptor (u-PAR) found in a variety of cells, is important for its activity. Plasmin generated from receptor bound u-PA plays a central role in the mediation of pericellular proteolysis, and is protected from rapid inactivation by its principal circulating inhibitor α2-antiplasmin (155).

Inhibition of fibrinolysis

α2-antiplasmin

α2-antiplasmin is the principal inhibitor of human plasmin (156). The 464 residue serpine is a glycoprotein with MW 67 kDa, and the plasma concentration is approximately 1 mM. The 464 residue α2-antiplasmin and a shorter form containing 453 amino acids are

present in plasma in about equal concentrations, and both form stable complexes with plasmin (157). Through interaction with the α2-antiplasmin reactive site located at Arg^{376}-Met^{377} plasmin forms a 1:1 stochiometric inactive complex (158). Plasmin not neutralized by α2-antiplasmin may be inactivated by circulating α2-macroglubuline (159).

Plasminogen activator inhibitor

In addition to inhibition of plasmin, fibrinolysis may also be inhibited at the activator level by plasminogen activator inhibitor 1 (PAI-1). It is a single chain, 379 residue glycoprotein with MW 52 kDA belonging to the serpine family (160;161). The active site is located at Arg346-Met347. PAI-1 becomes activated through interaction with vitronectin. This interaction induces a conformational change and an elongation of the reactive center loop (RCL) of the molecule, thus enabling the active binding site for plasminogen activators (162). Active PAI-1 rapidly interacts with one- and two-chained t-PA and two-chained u-PA, thus efficiently preventing fibrinolysis. In addition to its principal role as inhibitor of activators of fibrinolysis, PAI-1 has been shown to participate in angiogenesis.

Thrombin-Activatable Fibrinolysis Inhibitor

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a single chain protein in the family of zinc containing metallocarboxypeptidases with MW 60 kDa. It is produced in the liver in its inactive form, TAFI, and found in plasma at a concentration of 75 nM. Following activation by thrombin, plasmin or trypsin, the active form, TAFIa, exerts it antifibrinolytic effect by cleaving off C-terminal lysine residues from partially degraded fibrin. The lysine residues stimulate t-PA mediated plasmin activation. TAFIa, thus, prevents additional binding of plasminogen to the clot and inhibits further fibrinolysis (163;164).

Fibrinogen assays

Different techniques have been employed to determine the fibrinogen concentration in plasma. The method described by Jacobsson in 1955 has often been regarded as a reference method, reflecting the total clottability of fibrinogen (165). The fibrinogen concentration is measured after adding thrombin to a buffer diluted plasma sample, leaving the sample to clot for 2 hours, with subsequent syneresis and washing of the clot, and measuring the total clottable protein content spectrophotometrically after dissolving the clot in urea. The method is laborious and time consuming, and is not easily automated. Thus, it is not commonly used as a routine method. An assay based on the clotting rate of a diluted plasma sample was described by Clauss in 1957 (166), and implies the addition of a large quantity of thrombin to a diluted plasma sample, and recording the time to clot formation. Due to the high thrombin concentration, fibrin monomers form rapidly, and the method reflects the fibrinogen concentration and the polymerization properties of the fibrin monomers. The coagulation endpoint can be assessed manually as elapsed time to the formation of a visual clot, but clotting rate assays are also easily automated, making them suitable for routine purposes.

Other methods, such as salt or heat precipitation (167-169), are less commonly used as routine assays. Immunochemical assays are based on polyclonal antibodies reacting with epitopes on the fibrinogen molecule (167;170). The light scatter of insoluble antigen-antibody complexes correlates to the fibrinogen concentration in the sample. Other immunological methods include fibrinogen quantification using ELISA techniques. The variety of methods used to determine plasma fibrinogen levels, in addition to heterogeneous calibration standards, have made comparison of fibrinogen levels obtained from different laboratories difficult (171). In order to obtain comparable estimates of fibrinogen concentrations, the First International Fibrinogen Standard (IS) (89/644) was introduced by the National Institute for Biological Standards in 1992 (172), and replaced in 2000 by the 2nd IS for fibrinogen (173). The freeze-dried calibration plasma has enabled laboratories and manufacturers to calibrate against a common international fibrinogen standard.

Fibrinogen interaction with blood platelets and leukocytes

Through interaction with specific cell surface integrin receptors, fibrinogen may interact with blood platelets and leucocytes. Integrins are transmembrane, heterodimeric glycoprotein receptors, and their main tasks include coordination of cell adhesion to extra cellular matrix and cell to cell interactions (174). After activation by agonists such as ADP, collagen or thrombin the platelets become activated and display the GP IIb/IIIa integrin (α2bβ3), allowing fibrinogen-platelet interaction. Fibrinogen is the predominant ligand for activated platelets at low shear rates, whereas vWF is the physiologically important ligand at high share rates (175;176). The platelet-fibrinogen interaction is closely regulated to prevent spontaneous intravascular aggregation with potential deleterious effects. The IIb/IIIa receptor, therefore, is not constitutively expressed on platelets. Although both the fibrinogen α-chain Arg-Gly-Asp (RGD)-sequences (Aα 95-97 and 572-574) and the C terminal γ-chain (γ 400-411) are potential ligands for GP IIb/IIIa, fibrinogen-dependent platelet aggregation requires an intact C-terminal γ-chain. Thus, the alternatively spliced γ-chain, γ`, does not support platelet aggregation (177). The α-chain RGD sequences are neither necessary nor sufficient for platelet aggregation (178). Fibrinogen may also interact with the leukocyte integrin $\alpha_M\beta2$ (CD11b/CD18, Mac-1). The fibrinogen γ- chain region (γ 377-395 and γ 190-202) functions as ligands for the leukocyte $\alpha_M\beta2$ in vivo (179;180). Thus, fibrinogen binds to activated leucocytes, in the presence of divalent cations, with the association of about 150 000 fibrinogen molecules per cell (181). Previous studies have shown that fibrin(ogen) may have different effects on leukocyte function (182-184), and fibrin is involved in monocyte adhesion to endothelium and migration at sites of inflammation (185;186). Furthermore, fibrinogen plays an important role in the inflammatory response to implanted biomaterials (187;188). In addition to platelets and leucocytes, fibrinogen may also interact with fibroblasts, endothelial- and, epithelial cells (189;190).

5. AIMS OF THE STUDY

- To investigate the effect of freeze-drying on the clotting properties of fibrinogen, and how the use of a freeze-dried calibration standard might affect the results of a commonly used clotting rate fibrinogen assay.

- To determine whether the fibrinogen concentrations obtained by a clotting rate assay deviated from levels found using a method based on total clottability during acute-phase conditions, and to investigate if a discrepancy between the two methods correlates with changes in the HMW-fibrinogen fraction.

- To determine the influence of the major fibrinogen subfractions on the viscosity of native fibrinogen, and investigate the effect of EDTA on the viscosity of purified fibrinogen and plasma.

- To study the effects of moderate red wine consumption on plasma viscosity, fibrinogen concentration, and the distribution of the 3 major fibrinogen subfractions.

- To investigate potential proinflammatory effects of purified fibrinogen and fibrin on isolated peripheral blood mononuclear cells (PBMC) as reflected by increased mRNA expression and synthesis of the proinflammatory cytokines IL-6, TNF-α and IL-1β, and to assess the significance of an altered HMW/LMW-fibrinogen ratio.

6. MATERIALS AND METHODS

Study subjects

In paper I blood samples drawn from 14 healthy subjects (7 men, 7 women, and median age 22) were used to determine the fibrinogen concentrations in plasma before and after freeze-drying. Blood samples for the preparation of fresh-frozen and freeze-dried calibration standards were collected from 20 healthy subjects (10 men, 10 women, median age 22). The fibrinogen concentration in blood samples from 30 apparently healthy subjects (15 men, 15 women, median age 24) and 10 patients undergoing major elective surgery (4 men, 6 women, median age 66) were used to determine any discrepancy in fibrinogen concentration using the fresh frozen calibration standard compared to the freeze-dried standard.

In paper II the fibrinogen concentrations and the distribution of fibrinogen subfractions in 7 patients about to undergo elective surgery were studied (4 men, 3 women, median age 66 years). Three patients were undergoing total hip joint replacement, 2 had an explorative laparotomy, and 2 patients were undergoing hemicolectomy.

In paper IV we studied the effect of moderate red wine on fibrinogen levels, the distribution of fibrinogen subfractions and plasma viscosity in 92 healthy, nonsmoking men (35%) and women (65%) (mean age 50.3 years). Baseline characteristics of the study population are specified in paper IV.

In paper V we studied the effect of purified fibrinogen and HMW-fibrinogen on the synthesis of proinflammatory cytokines from isolated PBMC collected from 9 healthy subjects (5 women, 4 men, median age 31 years).

Fibrinogen assays

Total clottable protein assay

The total clottable protein assay was performed according to a modification of the method described by Jacobsson (165). Plasma was diluted to one fourth of its original concentration by adding phosphate buffer and thrombin (final concentration 7.5 NIH U/ml). After clotting for 30 min, the clot was synerized, washed in 0.9% saline and dissolved in 40% alkaline urea. The fibrinogen concentration was then calculated by measuring the absorption at 280 nm using the clot solvent as a blank, and correcting for extraneous absorption at 320 nm and 360 nm. The intra-assay coefficients of variation were 1.5% at a fibrinogen concentration of 3.6 g/l (n = 10) and 1.6% at a concentration of 4.7 g/l (n = 10).

Clotting rate assay

Fibrinogen concentrations in the experiments presented in paper II and III were determined using a clotting rate assay as described by Clauss (191). 200 ml of a solution containing 1 volume citrated plasma and 9 volumes sodium barbital buffer were incubated at 37 °C for 2 minutes. 100 µl thrombin 100 NIH U/ml was added, and the clotting time was determined. If the clotting time was shorter than 7 seconds, a higher dilution of plasma was used. If the clotting time exceeded 15 seconds, a lower plasma-dilution was used.

Automated clotting rate assay

Fibrinogen concentrations in the studies presented in paper I and IV were determined by and automated clotting-rate assay (Automated Coagulation Laboratory Futura®, Instrumentation Laboratory, Lexington, MA, USA) using the principle of turbidimetric clot detection. The intra-assay CV for the clotting-rate assay was 3.2% (n=10)

Freeze-drying

Freeze-drying of plasma samples (1 ml in each sample) was performed by The Axis-Shield company (Oslo, Norway), based on international guidelines (192), using a Gambolt GT 150 freeze dryer. The samples were sealed under vacuum, stored in small glass ampoules at -20 °C and reconstituted by adding 1 ml of purified water before use. Residual humidity in freeze-dried plasma samples was 0.4–0.6%.

Thrombin clotting time

0.1 ml of 3 IU/ml thrombin was added to 0.2 ml plasma or fibrinogen solution, incubated at 37 °C and the clotting time recorded.

Electrophoresis

Gels (3% polyacrylamid and 0.5% agarose) were prepared as described by Weinstein and Deykin (193). The electrophoretic procedure was performed using a Mini-PROTEAN® II (BioRad, Richmond, CA, USA). The gels were Coomassie-stained, and the fibrinogen fractions quantified by densitometric scanning using Kodak image station 440 CF with Kodak 1D image analysis software. The intra-assay coefficient of variation for the electrophoresis and densitometric scanning was 1.8% (n =10).

Fibrinogen purification

Fibrinogen used in paper III was purified from plasma donated by healthy volunteers using the β-alanine precipitation method described by Jacobsen and Kierulf (194), except that citrate (final concentration 0.011 M) was present during the entire procedure. Trasylol (final concentration 200 KIU/ml) and soybean trypsin inhibitor (STI, final concentration 0.1 mg/ml) were used as inhibitors instead of Epsilon aminocaproid acid.

Separation of HMW-, LMW- and LMW`-fibrinogen

Separation of the fibrinogen fractions was performed by gradual precipitation of a purified fibrinogen solution. A saturated ammonium sulphate solution was added slowly during constant stirring. The HMW-fraction was prepared by the addition of saturated ammonium sulphate to 19% saturation. After centrifugation at 1200 g for 7 min the precipitate was collected, dissolved in 0.15 M NaCl at 37 °C, re-precipitated in ammonium sulphate (19%), leaving the precipitate to dissolve in 1/3 of its original volume and finally dialyzed. The LMW-fraction was obtained from precipitate formed at 22–24% ammonium sulphate saturation and the LMW`-fraction at 26–30% saturation. Precipitates were centrifuged, dissolved in 0.15 M NaCl at 37 °C and dialyzed. All fractions were dialyzed three times against 500 vol diemal buffered saline for 24 h.

Contamination with soluble fibrin and other low solubility complexes were removed by leaving the dialyzed fractions at + 4 °C for 24 hours and removing insoluble material after centrifugation. The purity of the fibrinogen fractions was determined by sodium dodecyl sulphate (SDS) gel electrophoresis. The HMW-fibrinogen fraction was contaminated with 1% LMW. The LMW-fraction contained 96% LMW- and 4% LMW`-fibrinogen, and the LMW`-fraction contained 90% LMW`-fibrinogen and 10% HMW- and LMW-fibrinogen. The thrombin clotting times were 15, 24 and 31 seconds for HMW-, LMW- and LMW`-fibrinogen, respectively. The clottability of HMW-fibrinogen was 100%, of LMW-96% and of LMW`-fibrinogen 76%.

Viscometry

The viscosity of fresh plasma, purified fibrinogen and fibrinogen subfractions were determined using Haake Microvisco® 2 viscometer (Haake Mess-Technik, Munich, Germany). A metal sphere of standard weight and size was inserted into a precision cut syringe containing the sample, and placed in a tilted position inside the viscometer containing two photo sensors. The time needed by the ball to pass between the two photo sensors was measured and the viscosity calculated. The intra-assay coefficient of variation for the viscometer was 0.4% (n=10).

Isolation of PBMC

Whole blood was drawn by clean venipuncture from healthy volunteers who had not used any medication two weeks prior to inclusion, and collected into lipopolysaccharide (LPS)-free Vacutainer® tubes (Cedex, France) containing 7 mM EDTA. PBMC were isolated from whole blood under sterile conditions using Lymphoprep® (Axis-Shield PoC , Norton, MA, USA). Cell cultures comprised 81% lymphocytes, (25, 75 percentiles: 69, 82%), 11% monocytes (11, 14%) and 8% granulocytes (7, 16%).

RNA isolation and mRNA quantification

PBMC were incubated with purified HMW- or unfractioned fibrinogen (final concentration 0.2 mg/ml or 0.5 mg/ml) and RPMI-1640 containing 25 mM HEPES and L-glutamine. Following centrifugation the cell pellets were lysed in 250 µl lysis/binding

buffer (MagNA Pure LC RNA Isolation Kit-High Performance) and stored at –70 °C until analyzed. Total RNA was isolated using a MagNA Pure robot (Roche Diagnostics, Basel, Switzerland) and the "RNA hp-cells" protocol. The amount of total RNA was quantified using the NanoDrop® spectrophotometer (NanoDrop technologies, Wilmington, DE, USA) as described in paper V. Detection of the relative change in gene expression in fibrinogen stimulated PBMC compared to controls normalized to an endogenous reference (GAPDH mRNA) was estimated using the Comparative C Method of relative quantification (ΔΔCt method) (195).

Cytokine analysis

Concentrations of IL-6, TNF-α and IL-1β were determined in cell-lysate using commercial enzyme linked immunosorbent assays (ELISA) from R&D Systems (Minneapolis, MN, USA). Measurements were done in duplicates according to the manufacturer's instructions and the mean concentrations were calculated. The intra-assay CVs for the IL-6 cytokine assay were 1.7% at low (16.8 pg/ml) and 4.4% at high (191 pg/ml) concentrations, whereas the inter-assay CVs were 2.0% and 3.7%, respectively. Intra-assay CVs for the IL-1β assay were 2.4 at low (12.6 pg/ml) and 3.4% at high (192 pg/ml) concentrations, and the inter-assay CVs 3.4% and 7.1%, respectively. For the TNF-α assay the intra-assay CVs were 5.3% at low (2.4 pg/ml) and 8.8% at high (14 pg/ml) concentrations, and the inter-assay CVs 10.8% (2.4 pg/ml) and 16.7% (14 pg/ml), respectively.

Statistics

Normally distributed data were reported as mean ± standard deviation (SD). Comparison of data sets were valuated using Student`s t-test, and reported as the mean difference with 95% confidence interval (paper IV). Correlations between normally distributed variables were determined using Pearsons`s method.

If not normally distributed, data were presented as medians with range or the 25, 75 percentile. Comparisons between data sets were made using Wilcoxon signed rank test (paper I, II, III, V), and correlations between variables performed using Spearman`s rank correlation test. P values are two sided, and values less than 0.05 were regarded as

statistically significant. Statistical analyses were performed using the Statistical Package for Social Sciences version 11.0 and SamplePower 2.0 (SPSS Inc., Chicago, Illinois, USA).

7. SUMMARY OF RESULTS

I

Influence of freeze-drying on the clotting properties of fibrinogen in plasma

In paper I we studied the effect of freeze-drying on the clotting properties of fibrinogen. Freeze-dried plasma standards are commonly used to calibrate fibrinogen assays. Altered clotting properties following the freeze-drying process might influence the results obtained when using clotting rate assays to determine fibrinogen concentrations. To evaluate the effect of freeze-drying, we determined the fibrinogen concentrations in citrated plasma samples using a total clottable protein method and a clotting rate assay before and after freeze-drying. When using the clotting rate assay, significantly higher fibrinogen concentrations were found in fresh-frozen plasma samples compared to freeze-dried samples ($P<0.001$). In freeze-dried plasma samples, the fibrinogen concentrations were significantly higher using the total clottable protein assay than the clotting rate assay ($P<0.001$). When measuring the fibrinogen concentrations in plasma samples with a wide range of fibrinogen concentrations using the clotting rate assay, significantly higher fibrinogen concentrations were found using the freeze-dried calibration plasma, than the fresh-frozen calibration plasma ($P=0.02$). The results indicate that the clotting rate of fibrinogen in citrated plasma is reduced following freeze-drying. When applying freeze-dried calibration plasma in a clotting rate assay, higher fibrinogen concentrations are obtained than by using fresh-frozen plasma.

II

Discrepancy between fibrinogen concentrations determined by clotting rate and clottability assays during the acute-phase reaction

Little is known about the influence of the acute-phase reaction on clotting rate assays. In this paper we wanted to investigate discrepancies between fibrinogen concentrations obtained by a clotting rate assay (as described by Clauss) and a total clottable protein assay (according to Jacobsson). We compared the fibrinogen concentrations using the two methods, and determined the HMW (High Molecular Weight)-, LMW- and LMW`-fibrinogen fractions in plasma samples collected preoperatively and on postoperative days 1, 3, and 5 in patients undergoing major elective surgery. Good agreement between

the two assays was found in samples drawn before the operation. In samples collected on postoperative days 1 and 3, the fibrinogen concentrations determined by the clotting rate assay were significantly higher than the concentrations measured with the total clottable protein assay ($p = 0.015$ on both days). SDS-gel electrophoresis showed an increase in the median HMW-fraction from 69.7% (range 64.3-70.4) in preoperative samples to 85.8% (80.7-87.6) in samples drawn on day 3. The difference between fibrinogen concentrations obtained by the two methods was significantly correlated to the HMW-fraction of the samples ($r=0.81$, $p < 0.0001$).

III

The viscosity of fibrinogen subfractions and of EDTA denatured fibrinogen do not differ from that of native fibrinogen

Fibrinogen is a major determinant of plasma viscosity. It has been suggested that the increased risk of cardiovascular disease associated with a high fibrinogen concentration may partly be attributed to its effect on viscosity. In paper III we wanted to evaluate how the three fibrinogen subfractions high molecular weight (HMW)-, low molecular weight (LMW)-, and very low molecular weight (LMW`)-fibrinogen affect viscosity. Furthermore, the viscosity of plasma is usually determined in ethylenediaminetetraacetic acid (EDTA) anticoagulant plasma samples. EDTA affects the clotting properties of fibrinogen due to denaturation, which might also affect the viscosity of fibrinogen. Thus, we also wanted to investigate the effects of EDTA on the viscosity of fibrinogen. Purified fibrinogen was obtained by beta-alanine precipitation of plasma from healthy donors. Separation of the fibrinogen fractions was performed by gradual precipitation of purified fibrinogen by ammonium sulphate. The viscosity was determined using a Haake Microvisco 2 viscometer. The viscosity of the three fibrinogen subfractions did not differ significantly from that of native fibrinogen. A significant prolongation of the thrombin clotting time was observed in the fibrinogen solution containing EDTA at 37 °C compared to 20 °C. However, the viscosity of EDTA anticoagulated purified fibrinogen and plasma samples did not differ from that of heparin anticoagulated samples.

IV

A daily glass of red wine induces a prolonged reduction in plasma viscosity: a randomized controlled trial.

Moderate red wine consumption has been linked to reduced risk of coronary heart disease. Reduced plasma viscosity and reduced fibrinogen levels have been launched as possible contributors to this risk reduction. In study IV we investigated the effect of moderate red wine consumption on plasma viscosity, fibrinogen concentration and fibrinogen subfractions. We recruited 92 healthy, nonsmoking volunteers to consume one glass of red wine daily for 3 weeks in a prospective, randomized cross-over study. In the second 3-week period the volunteers abstained from alcohol use. The plasma viscosity, fibrinogen concentration and the distribution of the main fibrinogen subfractions were determined at inclusion, after wine drinking and after abstention. Plasma viscosity was reduced by 0.026 and 0.024 mPa.s in the two groups following wine intake (95% CI 0.009-0.043, p=0.004/0.0083-0.039, p=0.003). The decrease in plasma viscosity was maintained following 3 weeks of abstention. The fibrinogen concentration was reduced by 0.17 g/l following wine drinking in the group starting with abstention (CI 0.04-0.29, p=0.01). The distribution of the fibrinogen subfractions remained unaltered. The decreased viscosity levels were maintained after 3 weeks of abstention, suggesting a sustained viscosity lowering effect of red wine.

V

Fibrinogen and fibrin induce synthesis of proinflammatory cytokines from isolated peripheral blood mononuclear cells

Fibrinogen in plasma includes three main fractions; high-molecular-weight (HMW) -fibrinogen, low-molecular-weight (LMW) -fibrinogen, and very-low-molecular-weight (LMW`) -fibrinogen. During acute-phase conditions, plasma fibrinogen levels and the HMW-/LMW-fibrinogen ratio increase rapidly due to increased synthesis of HMW fibrinogen. The consequences of elevated plasma fibrinogen levels and local deposition of fibrin in inflammatory tissues observed during acute-phase conditions are not clear. We wanted to investigate proinflammatory effects of fibrinogen and fibrin on peripheral blood mononuclear cells (PBMC) as reflected by altered mRNA expression and synthesis

of the proinflammatory cytokines IL-6,TNF- α and IL-1β , and to explore the significance of altered HMW-/LMW-fibrinogen ratio. PBMC were isolated from whole blood using Lymphoprep®. HMW-fibrinogen was separated from unfractioned fibrinogen by ammonium sulphate precipitation. Cells were incubated with unfractioned fibrinogen, HMW-fibrinogen or fibrin. Cytokine levels in cell lysates were determined using ELISA assays. Real-time PCR was used for mRNA quantification. We found that fibrinogen significantly increased mRNA levels, and induced synthesis of the proinflammatory cytokines IL-6 and TNF- α in PBMC in a dose dependent manner. Median (25, 75 percentile) IL-6 and TNF- α concentrations were 12 (5, 40) pg/ ml and 16 (0,61) pg/ml, respectively. Median mRNA quantity was increased 12.3– (6.6, 48.6) and 1.7– (1.5, 6.5) fold for IL-6 and TNF- α compared to controls. The stimulatory effect of unfractioned fibrinogen was not significantly different from HMW-fibrinogen. Fibrinogen and fibrin were equally effective in promoting cytokine synthesis from PBMC.

8. GENERAL DISCUSSION

Paper I

In 1991 the standardization and scientific committee of the International Society on Thrombosis and Haemostasis (ISTH) supported the establishment of a lyophilized fibrinogen standard (coded 89/644) as an international standard for fibrinogen. The lyophilized plasma was manufactured by The National Institute for Biological Standards and Control (UK) and comprised lyophilized material from a plasma pool of 25 healthy donors (196). The fibrinogen concentration was determined by 22 different laboratories, and the declared concentration of 2.4 g/l clottable protein was the geometric mean of the reported values from the participating laboratories. A total clottable protein method was the recommended assay to determine the fibrinogen concentration in the standard. The use of a common calibration standard is essential to obtain accurate and comparable results between laboratories. Previous studies have indicated that the declared value of standards in commercial available fibrinogen kits may vary by as much as 200% (197). In paper I we investigated the effect of freeze-drying of plasma samples on the fibrinogen concentrations obtained by an modified automated Clauss assay compared to a total

clottable protein according to Jacobsson (165). The total clottable protein assay yielded similar results in fresh frozen and freeze-dried samples. When using the clotting-rate assay, however, significantly higher fibrinogen concentrations were found in fresh-frozen samples compared to those found in freeze-dried specimens (mean difference 0.18 g/l, 95% confidence interval 0.13– 0.23, $P < 0.001$). The precision of both fibrinogen assays were evaluated, and given as the percent coefficient of variation (CV). The intra-assay CVs of the fibrinogen assays used were less than 2 % at the fibrinogen concentrations found in our samples, which indicate a high precision. Although inter-assay CVs were not determined, between-days variability was reduced by performing all comparisons between the two assays on the same day and by the same operator. Freeze-drying has been suggested to induce changes in the secondary and tertiary structure of proteins (198;199). This denaturation might affect its physical properties and biological activity. As the principal function of fibrinogen is clotting, the freeze drying process may inflict conformational changes in the native structure which might affect its clotting properties. Reduced clottability has been considered as evidence of denaturation of fibrinogen. Ly and coworkers suggested, however, that the thrombin clotting time might be a better indicator of denaturation than the clottability (200). They also found that the denaturation process did not reduce the fibrinogen N-terminal susceptibility to thrombin. This may imply that denaturation primarily affects the polymerization properties of the fibrin monomers, thus, influencing assays based on clotting rate to a larger extent than assays reflecting the total clottability of fibrinogen. The use of diluted plasma and large amounts of thrombin in the clotting rate method used in our study makes this assay even more sensitive to polymerization defects than the thrombin clotting time. The freeze-drying process may also alter the turbidity of the plasma samples (173), which may induce discrepancies in results obtained by the two assays. The fibrinogen concentration is calculated after spectrophotometrically measuring the absorption at 280 nm and correcting for extraneous absorption at 320 nm and 360 nm. Samples with optical densities (OD) at 320 nm exceeding 10% of OD 280 may underestimate the fibrinogen concentration. Freeze drying of the plasma samples had little effect of the turbidity in our study ($OD_{320} < 10\%$ of OD_{280}), and the freeze drying process did not affect the results obtained by the clottability assay compared to fresh frozen samples. To investigate the

effect of using a freeze-dried plasma standard compared to a fresh-frozen standard, the fibrinogen concentrations in plasma samples from individuals with a wide range of fibrinogen concentrations were determined using the clotting-rate assay. Significantly higher fibrinogen concentrations were obtained using the freeze-dried calibration plasma compared to corresponding fresh-frozen plasma (mean 0.04 g/l, 95% CI 0.006-0.08, p=0.02). The difference is small, however, and probably negligible in every-day clinical practice. In epidemiological studies, however, when comparing minor differences in fibrinogen concentrations, the influence of using freeze-dried calibration plasmas should be taken into consideration. The 1st fibrinogen standard was replaced in 2000 by the 2nd International Standard for Fibrinogen (coded 98/612) prepared by the UK NIBSC and recommended by the ISTH fibrinogen subcommittee. The fibrinogen content of this standard was determined using an automated clotting-rate assay (173).

Paper II

In paper II we compared the plasma fibrinogen concentrations in patients undergoing major elective surgery obtained by a commonly used clotting rate assay (166), with a total clottable protein assay according to a modification of the method described by Jacobsson (165). Holm and Godal had previously shown that the HMW-/LMW-fibrinogen ratio increases rapidly during acute-phase conditions such as myocardial infarction and major surgery (201), and that the thrombin clotting times of LMW- and LMW`-fibrinogen were prolonged compared to HMW-fibrinogen due to altered polymerization properties of the 3 fibrinogen fractions (63). The clotting rate assay procedure implies the addition of a high concentration of thrombin (final concentration 33 NIH U/ml), with a subsequent rapid release of fibrinopeptide A (FPA), resulting in a clotting rate depending on fibrinogen concentration and the polymerization properties of the fibrin monomers. A high correlation between the divergence in fibrinogen concentrations obtained by the two methods and the HMW-fibrinogen level was found (r = 0.81), indicating that the increase in HMW-fibrinogen may be an important contributor to the observed discrepancy.

Both fibrinogen assays used in the study have been extensively validated previously (165;166), and the precision of the two assay was good (intra assay CVs were 1.2-3.2% and 1.5-1.6% for the clotting rate and the clottability assays respectively). All samples

from each patient were assayed on the same day and by the same operator, thus reducing between-days variability. Acute-phase conditions affects the phosphorylation of FPA, which may increase from the normal 25-30% to over 50% (141). This has been suggested to affect the release rate of FPA. However, a more effective release of phosphorylated FPA than of FPA would probably have a limited effect on our results since high thrombin concentrations are added to diluted plasma. The blood samples were obtained from non-fasting patients. Although it is clear that lipoproteins are included in fibrin clots obtained from lipemic plasma (165), the error in fibrinogen determination of lipemic plasma samples is small, providing that correction for "extraneous absorption" is made (165). All clottability measurements in our study were done at 280 nm wavelength, correcting for extraneous absorption at 320 and 360 nm. Furthermore, all plasma samples were visually inspected and none found to be lipemic.

The results illustrate that variations in the HMW-/LMW-fibrinogen ratio may affect results obtained by a clotting rate assay, and contribute to discrepancies between methods reflecting the total clottability of fibrinogen and assays reflecting the functional state of fibrinogen.

Paper III

In paper III we investigated the influence of the 3 major fibrinogen subfractions on the viscosity of native fibrinogen, and found that the viscosity of high molecular weight (HMW-) fibrinogen did not differ from that of low molecular weight (LMW)-, or very low molecular weight (LMW`) fibrinogen. This implies that degradation of the C-terminal end of one- (LMW-fibrinogen) or both (LMW`-fibrinogen) α-chains of the fibrinogen molecule, representing a loss in molecular weight of 35 kDa (10% of native weight) and 70 kDa (20 % of native weight,) respectively, does not significantly reduce the viscosity of fibrinogen. Fibrinogen is a major determinant of plasma viscosity (134;135). Thus, altered plasma viscosity attributable to increased fibrinogen levels in acute-phase conditions is not related to concomitant changes in the distribution of the major fibrinogen subfractions. At equal concentrations the size and shape of proteins determine their effect on plasma viscosity. Normally, the αC domain of the fibrinogen molecule extends from the D region, parallel to the coiled-coil of the Aα-, Bβ- and γ-

chains, interacting with the central region of the molecule or forming intra-molecular αC-αC connections (29;30). Following cleavage of FPB the αC domains dissociate from the central region and become available for inter-molecular αC-αC connections (30). The lack of effect on fibrinogen viscosity following the loss of the αC-domains may indicate that the αC-domain is of minor importance to the overall shape of the fibrinogen molecule. Potential sources of error in this study comprise impurities of the isolated fibrinogen fractions. Separation of the fibrinogen fractions was performed by gradual precipitation of a purified fibrinogen solution, and the purity of the subfractions determined by sodium dodecyl sulphate (SDS) gel electrophoresis. The HMW-fibrinogen fraction was contaminated with 1% LMW. The LMW fraction contained 96% LMW- and 4% LMW'-fibrinogen, and the LMW' -fraction contained 90% LMW'-, and 10% HMW- and LMW-fibrinogen. The reason why the LMW`-fibrinogen contained HMW-fibrinogen and the LMW-fibrinogen fraction did not is unclear. Possibly, the higher ammonium sulphate concentration needed to precipitate the LMW`-fibrinogen fraction may have promoted a more effective precipitation of the remaining HMW-fibrinogen in the solution. Contamination of the fibrinogen solutions with soluble fibrin and other low solubility complexes were reduced by leaving the dialyzed fractions at 4 °C for 24 h and removing insoluble material following centrifugation. Remaining contaminations with proteins such as fibronectin represent potential sources of error. Fibronectin is a common contaminant in purified fibrinogen solutions, and it is up-concentrated during the fibrinogen precipitation procedure (202). The fibronectin concentrations in the fibrinogen subfractions in our study were low; 1.8 μg/g fibrinogen (HMW fraction), 6.0 μg/g fibrinogen (LMW fraction) and 26 μg/g fibrinogen (LMW` fraction). Furthermore, the specific viscosity of fibrinogen is significantly higher than that of other plasma proteins. Thus, protein contamination of the LMW`-fibrinogen fraction would reduce the viscosity. No differences in viscosity levels in the 3 fibrinogen fractions were observed in our study. The intra-assay CV for the quantification of the fibrinogen fractions was 1.8%, indicating a high precision for the electrophoresis and densitometric scanning procedures. Adding EDTA, a commonly used anticoagulant, to purified native fibrinogen did not affect the viscosity despite a significant increase in thrombin clotting time. EDTA is a chelating agent which effectively removes calcium. Calcium ions are essential to preserve

the structure and function of the fibrinogen molecule (200;203;204). Removal of calcium facilitates denaturation of fibrinogen as reflected by increased thrombin clotting time (33;200). Denaturation of proteins has been closely associated with changes in viscosity (205), but the denaturation of fibrinogen induced by EDTA does not significantly affect the viscosity of plasma compared to heparin anticoagulated plasma.

Paper IV

In paper IV we wanted to investigate the effect of a daily glass of red wine on plasma viscosity, fibrinogen levels and the distribution of the major fibrinogen subfractions. Moderate alcohol consumption has been linked to reduced risk of ischemic heart disease (206-208). A beneficial effect on haemostatic factors such as fibrinogen and plasma viscosity has been launched as possible contributors to this risk reduction. The study was a prospective, randomized crossover trial, and a sub-study of the work by Retterstøl and colleagues from 2005 (209). The study recruited 92 healthy men (35%) and women (65%). Smoking significantly affects fibrinogen levels and plasma viscosity, and only non-smokers were recruited in the trial (210). The crossover design implies that the volunteers were randomized to start with 3 weeks of red wine drinking or abstention, and subsequently crossed over to the other intervention. This design is particularly effective in intervention studies, because it enables each individual to be its own control, and, thus, eliminates between-subject variation. This increases the statistical power of the study compared to parallel group designs, and requires a smaller sample size. Crossover studies are, however, sensitive to carryover effects. As illustrated in figure 1 and 2 in paper IV, the observed mean plasma viscosity and fibrinogen concentration following the abstention period differed in the subgroups, indicating a prolonged effect of wine drinking on plasma viscosity. This effect and the fact that the majority of the participants were moderate alcohol consumers at the time of inclusion (median consumption 1-3 units per week) may explain why no difference in plasma fibrinogen levels were observed in the group starting with wine drinking. The prolonged effect of wine drinking on plasma viscosity was an unexpected but interesting finding in the study, which, to our knowledge, has not previously been discussed. Including abstention periods at the beginning of the study and between each treatment period, might have reduced this

effect. Due to the prolonged viscosity lowering effect of red wine, the results from each subgroup were analyzed and presented separately. For logistical reasons, the alcohol intake in the study was not supervised. Although the importance of complying with the study protocol was carefully explained to the participants, lack of adherence to the scheduled intervention represents a potential confounder in the study. Self reported compliance was, however, good. Five participants reported missing 1-2 days in the wine period, and two participants consumed 1-2 units of alcohol during the abstention period, which may have attenuated the effects on viscosity and fibrinogen levels, and potentially reduced the observed differences between the wine drinking- and abstention periods.

Although the participants were encouraged to maintain a normal diet, data concerning diet and physical exercise were not recorded. However, only ethanol consumption seems to have a clear and consistent effect on plasma fibrinogen levels. Other dietary components seem to have modest or no effects (for review see (211)). Thus, changes in diet during the study period do probably not represent a potential confounder on fibrinogen levels. Blood sampling were performed in non-fasting subjects. The effects of meals seem to have little effect on plasma viscosity measurements, however (212;213).

Plasma viscosity was determined by a “falling ball” viscometer. The intra assay CV of the assay was 0.41%, indicating a high precision in measurements. Blood sampling and measurements were performed according to international guidelines, except that frozen plasma samples were used. No statistically significant differences in viscosity between corresponding fresh and thawed frozen plasma samples were found in our study. Differences in study populations, smoking and alcohol consumption, fibrinogen levels and different assays used for viscosity determination, may contribute to differences in observed plasma viscosity in different trials (214-216). Increased plasma viscosity and fibrinogen levels have been identified as independent markers of increased risk of CHD (217). In addition to raising plasma high density lipoprotein (HDL) levels, reduced plasma viscosity and fibrinogen levels may contribute to the observed health benefits associated with moderate red wine consumption.

Paper V

Close interaction exists between the inflammatory- and coagulations systems. Thus, local and systemic inflammation may induce initiation of the coagulation system, and activation of coagulation may, on the other hand, affect inflammatory activity (for review see (218)). Tissue factor (TF) plays a central role in the inflammation induced activation of coagulation, but the interactions between the two systems also include thrombin, protein C and the fibrinolytic system. On the other hand, proinflammatory cytokines and chemokines have been shown to promote the expression of TF and induce coagulation (219).

In paper V we found that purified fibrinogen and fibrin significantly upregulated mRNA and synthesis of the proinflammatory cytokines IL-6 and TNF-α in isolated peripheral blood mononuclear cells (PBMC). Fibrinogen and fibrin were equally effective, and no difference in stimulatory effects were found between unfractioned fibrinogen containing 59% HMW-, 39% LMW- and 2% LMW`-fibrinogen and purified HMW-fibrinogen containing 99% HMW- and 1% LMW-fibrinogen, suggesting that the C-terminal α-chain is not essential for the stimulatory effects. Altered HMW-/LMW ratios, thus, seem to be of minor importance for the potential proinflammatory effects of fibrinogen/fibrin. Higher fibrin(ogen) concentrations in the cell cultures yielded higher cytokine levels in the supernatant, indicating a dose dependant effect.

Previous reports have indicated that fibrinogen may affect leukocyte function (182;183;220-224), and Perez and coworkers found that fibrin and fibrinogen may induce synthesis of IL-1β from PBMC (184). The integrins CD11b/CD18 (Mac-1) and, to a less extent, CD11c/CD18, seem to be the principal fibrinogen receptors on monocytes, macrophages and lymphocytes. The fibrin(ogen) γ-chain ($\gamma^{190\text{-}202}$ and $\gamma^{377\text{-}395}$) contains the putative recognition sites for the integrins (225). Fibrinogen mediated activation of transcriptional factors such as NF-kappa B have been implicated in the activation process (226;227). Circulating PBMC are continuously exposed to fibrinogen in vivo without producing significant levels of proinflammatory cytokines in healthy individuals (228). Thus, cell activation seems to be required for the cells to express CD11b/CD18 and thus facilitate cell-fibrinogen interaction (228;229).

We used cultured PBMC isolated from whole blood to study the effects of fibrinogen and fibrin. PBMC includes monocytes and lymphocytes and are commonly used to study the effect of stimulants ex vivo. The advantage of using isolated cell cultures is that the effects of stimulatory or inhibitory agents on specific cells can be studied in detail. Since cytokines are mainly released and consumed locally at the site of inflammation, cytokine levels in peripheral blood often do not reflect local inflammation and, thus, can not be used to study local inflammatory mechanisms. The disadvantages of using isolated cell cultures include removal of cytokines and other stimulatory or inhibitory factors during the isolation process. These factors may play essential roles in the inflammatory response in vivo. Furthermore, the isolation process and subsequent incubation may in it self activate the cells and make them more susceptible to stimulants (230). Therefore, inflammatory responses to stimulants observed in isolated cell cultures do not necessarily reflect the physiological responses in vivo. Whole blood systems may be better in reproducing the natural cell environment, allowing interaction between different cells, and including natural occurring stimulatory or inhibitory factors. Several potential confounding factors may have affected the results from our study. Cell activation and synthesis of proinflammatory cytokine due to lipopolysaccarids (LPS) or bacteria contamination of collection tubes, reagents and buffers during the blood sampling or cell isolation process represent an important potential source of error when studying inflammatory responses in cell cultures (231-233). Blood samples in our study were drawn from apparently healthy subjects who had not used any medication two weeks prior to blood sampling. We used sterile, endotoxin tested sampling tubes and buffers, and the PBMC isolation procedure was performed under strict sterile conditions. When present, LPS contamination was removed using an endotoxin removal resin (End-X® B-52, Associates of Cape Cod, Woods Hole, MA, USA). Before used, all buffers, culture media, electrolyte solutions and fibrinogen solutions were tested for LPS contamination using a highly sensitive chromogenic end-point assay with a lower detection limit of 1 pg/ml LPS (0.01 EU/ml), and deemed LPS free. LPS contamination below the detection limit of the assay, although not capable of inducing significant cytokine synthesis, may have contributed to cell activation. Following isolation, the cell cultures were incubated at 37 °C for 10 hours, before frozen at -70 °C. Activation of cells during the isolation

procedures and incubation period is well known. Previous trials have demonstrated changes in steady-state expression levels of cytokine mRNAs such as IL-1, IL-6, and TNF-α during culture of human PBMC, even under control conditions (230). Spontaneous in vitro aggregation or adherence to the culture wells might also promote activation and be of relevance in gene expression studies. Cytokine synthesis in our control cell cultures were either absent or weakly detectable. Importantly, however, all cells were exposed to the same isolating and incubation procedures, and supernatant from cells incubated with fibrinogen or fibrin contained significantly higher cytokine levels than controls. Furthermore, incubating cells with liquor from the defibrinated fibrinogen solutions (after clotting with thrombin) did not induce cell cytokine synthesis, indicating that fibrinogen indeed promoted mRNA upregulation and cytokine synthesis from the cells.

Variations in mRNA upregulation and cytokine production in cell cultures from different donors might be attributable to variations in activation or the number of monocytes in the cell cultures. All cell cultures contained significant numbers of granulocytes (25, 75 percentile: 7, 16%), which might also have affected our results. However, previous cell stimulation studies indicate a strong correlation between the production levels of proinflammatory cytokines like IL-1β, IL-6, and TNF-α and the number of monocytes, indicating that monocytes may be the principal source of synthesis of these cytokines in cell cultures (228).

Limited data exists on proinflammatory effects of fibrinogen and fibrin in vivo. It is clear that fibrinogen plays a central role in the inflammatory response to implanted biomaterials (187;188), and animal studies have suggested that local deposition of fibrin sustains inflammation in some inflammatory diseases (234). Due to the close interaction between fibrin(ogen) and inflammatory cells in acute and chronic inflammatory diseases in vivo (235-237), possible proinflammatory effects of fibrin(ogen) might modify the inflammatory responses in a wide range of inflammatory disorders. Further research is needed, however, to elucidate the interaction between fibrin(ogen) and inflammatory cells, and determine potential proinflammatory effects. Experiments should include whole blood systems as well as isolated cell systems including monocytes/macrophages and lymphocyte subclasses.

9. CONCLUSIONS

1. Freeze-drying of human plasma decreases its clotting rate. When using a clotting rate fibrinogen assay calibrated with freeze-dried plasma, higher fibrinogen concentrations are obtained compared to using fresh-frozen plasma (paper I).

2. Higher plasma fibrinogen concentrations are obtained using a clotting rate fibrinogen assay than a total clottable protein assay following major surgery. The discrepancy in results from the two methods is correlated to an increased HMW-fibrinogen fraction (paper II).

3. The viscosity of the 3 main fibrinogen subfractions HMW-, LMW-, and LMW`-fibrinogen do not differ from that of native fibrinogen. Adding EDTA to purified fibrinogen induces denaturation of the fibrinogen molecule as reflected by prolonged thrombin clotting time, but does not affect viscosity. The use of EDTA as plasma anticoagulant does not significantly affect plasma viscosity compared to Heparin anticoagulated plasma (paper III).

4. A daily glass of red wine induces a prolonged reduction in plasma viscosity. Fibrinogen levels are also reduced when preceded by an abstention period (paper IV).

5. Fibrinogen upregulates mRNA and synthesis of the proinflammatory cytokines IL-6 and TNF-α in isolated peripheral blood mononuclear cells. Fibrinogen and fibrin are equally effective in promoting cytokine synthesis, and altered HMW-LMW-fibrinogen ratio does not alter the proinflammatory effects (paper V).

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